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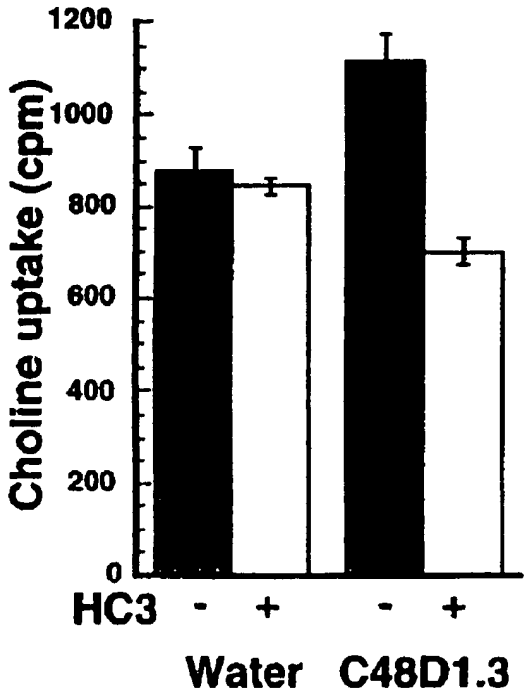

(57) Abrégé/Abstract:
A protein having a high affinity choline transporter activity which is important physiologically; a gene encoding the protein; and a method of screening a substance promoting the high affinity choline transporter activity with the use of the same, etc. The high affinity choline uptake activity of Na^{+}-dependent transporter cDNA deduced from the genomic sequence of a nematode (C. elegans) is examined in a Xenopus laevis oocyte expression system to thereby identify the cDNA (cho-1) of nematode high affinity choline transporter. By using the homology of a base sequence with this cDNA as an indication, the cDNA (CHT1) of rat high affinity choline transporter is cloned from rat spinal cord. Similarly, the cDNA of human high affinity choline transporter is cloned from human genome.

Abstract

The present invention provides a protein having high-affinity choline transporter activity which is important physiologically, a gene encoding the protein, and a method of screening a material promoting the high-affinity choline transporter activity with the use of the same, and the like. By examining high-affinity choline uptake activity of Na^+-dependent transporter cDNA deduced from the genomic sequence of a nematode (C.elegans) in a Xenopus oocyte expression system, the cDNA (cho-1) of nematode high-affinity choline transporter is identified. Then the cDNA (CHT1) of rat high-affinity choline transporter is cloned from rat spinal cord by using the homology of a base sequence to this cDNA as an index. Similarly, the cDNA of human high-affinity choline transporter is cloned from human genome.

SPECIFICATION

TITLE OF THE INVENTION

HIGH-AFFINITY CHOLINE TRANSPORTER

Technical Field

This invention relates to a protein having high-affinity choline transporter activity, a gene encoding said protein and the use of the same.

Prior Art

The autonomic nervous system which spreads to organs throughout a body and regulates the most basic functions of living organism including energy metabolism, circulation, respiration and reproduction along with endocrine system, is classified into the sympathetic and parasympathetic nervous systems. All autonomic nerve fibers excluding postganglionic fibers of the sympathetic nerve, motor nerve fiber, and sudoriferous gland/blood vessel dilative fiber in the sympathetic nerve are cholinergic, and acetylcholine is vital for the function of the autonomic nerve and the motor nerve. It has been known that the cholinergic neuron, being observed also in the brain, is important for recognizing function of the brain and that it degenerates after the onset of Alzheimer's disease. In the cholinergic neuron, because of lack of biosynthetic ability for choline, choline, an acetylcholine decomposition product, is taken up into a cell by a high-affinity choline transporter at the presynaptic terminals to be reused for synthesizing acetylcholine. The high-affinity choline uptake is a rate-limiting step for acetylcholine

synthesis and is presumed to regulate the efficiency of synaptic transmission (J. Neurochem. 18, 781-798, 1971, Science 178, 626-628, 1972, Biochem. Biophys. Acta 291, 564-575, 1973, Mol. Pharmacol. 9, 630-639, 1973, J. Pharmacol. Exp. Ther. 192, 86-94, 1975, J. Neurochem. 30, 15-21, 1978, J. Neurochem. 44, 11-24, 1985, J. Neurochem. 60, 1191-1201, 1993, J. Neurochem. 20, 581-593, 1973, Eur. J. Pharmacol. 102, 369-370, 1984). To date, most of cDNAs of transporters for major neurotransmitters have been isolated, however, a cDNA of the high-affinity choline transporter, which is physiologically important, has not been identified.

Disclosure of the Invention

So far, the existence of a protein being localized in the cholinergic neuron and having a function of taking up choline, a precursor of acetylcholine, into a cell has been expected, but molecular properties of said protein, a high-affinity choline transporter, have been unknown. An object of the present invention is to provide a physiologically important protein having the high-affinity choline transporter activity, a gene which encodes the protein, and a screening method of a high-affinity choline transporter activity promoter using the protein, the gene and the like.

The inventors have conducted intensive study to attain the above-mentioned object: with information of genomic project (Science 282, 2012-2018, 1998), Na^+-dependent transporter cDNAs being expected from the genomic sequence of a nematode (C. elegans) were cloned one by one, and the high-affinity choline uptake activity of each cDNA was examined in the oocyte expression system of Xenopus, and the cDNA of nematode

high-affinity choline transporter (cho-1) was identified on the basis of the above examination, then homologous molecules (CHT1) were cloned from rat spinal cord by using the homology of a base sequence to the cDNA as an index. This CHT1 had no homology to neurotransmitter transporters (J. Neurochem. 71, 1785-1803, 1998), but had 20 to 25% homology to molecules which belong to Na^+-dependent glucose transporter family (Nature 330, 379-381, 1987).

Northern blot analysis revealed that transcripts of CHT1 were confirmed only in spinal cord, basal forebrain, corpus striatum and brain stem, and CHT1 seemed to be expressed in cholinergic neurons. Accordingly, CHT1 was expressed in oocytes of Xenopus. As a result, choline uptake activity that is Na^+-dependent and completely inhibited by hemicholinium-3 was observed. These results indicate that CHT1 has high-affinity choline transporter activity. Further, the inventors have cloned choline transporter cDNAs derived from a human and from a mouse, and determined their base sequences, and have confirmed that their expression products have high-affinity choline uptake activity. The present invention has thus completed.

The present invention relates to a gene which encodes a protein having high-affinity choline transporter activity (claim 1), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 2, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having high-affinity choline transporter activity (claim 2), DNA containing a base sequence represented

by Seq. ID No. 1 or its complementary sequence and a part or a whole of these sequences (claim 3), DNA derived from a nematode which hybridizes with DNA comprising a gene according to claim 3 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 4), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 4, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having high-affinity choline transporter activity (claim 5), DNA containing a base sequence represented by Seq. ID No. 3 or its complementary sequence and a part or a whole of these sequences (claim 6), DNA derived from a rat which hybridizes with DNA comprising a gene according to claim 6 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 7), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 6, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having high-affinity choline transporter activity (claim 8), DNA containing a base sequence represented by Seq. ID No. 5 or its complementary sequence and a part or a whole of these sequences (claim 9), DNA derived from a human which hybridizes with DNA comprising a gene according to claim 9 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 10), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq.

ID No. 8, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.8, and having high-affinity choline transporter activity (claim 11), DNA containing a base sequence represented by Seq. ID No. 7 or its complementary sequence and a part or a whole of these sequences (claim 12), and DNA derived from a mouse which hybridizes with DNA comprising a gene according to claim 12 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 13).

The present invention also relates to a protein having high-affinity choline transporter activity (claim 14), a protein comprising an amino acid sequence represented by Seq. ID No. 2 (claim 15), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having nematode high-affinity choline transporter activity (claim 16), a protein comprising an amino acid sequence represented by Seq. ID No. 4 (claim 17), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having rat high-affinity choline transporter activity (claim 18), a protein comprising an amino acid sequence represented by Seq. ID No. 6 (claim 19), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having human high-affinity choline transporter activity (claim 20), a protein comprising an amino acid sequence represented by Seq. ID No. 8 (claim 21), and a protein comprising an amino acid

sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.8, and having mouse high-affinity choline transporter activity (claim 22).

The present invention further relates to a fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag (claim 23), the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 24), the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18 (claim 25), the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 26), and the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 27).

The present invention still further relates to an antibody which specifically binds to a protein having high-affinity choline transporter activity (claim 28), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 29), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has rat

high-affinity choline transporter activity according to claim 17 or 18 (claim 30), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 31), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 32), and the antibody according to any one of claims 28 to 32, wherein the antibody is a monoclonal antibody (claim 33).

The present invention also relates to a host cell containing an expression system which can express a protein having high-affinity choline transporter activity (claim 34), the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 35), the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18 (claim 36), the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 37), and the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 38).

The present invention further relates to a non-human animal in which function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or

overexpresses on its chromosome (claim 39), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 40), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18 (claim 41), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 42), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 43), and the non-human animal according to any one of claims 39 to 43, wherein the non-human animal is a mouse or a rat (claim 44).

The present invention still further relates to a preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene or the DNA according to any one of claims 8 to 10 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome (claim 45), the preparing method of a cell having high-affinity choline transporter activity according to claim 45, wherein the cell having high-affinity choline transporter activity is integrated with the gene or the DNA according to any one of claims 8 to 10 in its chromosome, and stably shows high-affinity choline transporter activity (claim 46), and a cell having high-affinity choline transporter activity being obtainable by

the preparing method of a cell having high-affinity choline transporter activity according to claim 45 or 46 (claim 47).

The present invention also relates to a screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in measuring/evaluating high-affinity choline transporter activity of the protein having high-affinity choline transporter activity according to any one of claims 14 to 22 in the presence of a subject material (claim 48), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated (claim 49), the screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 49, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the host cell containing an expression system which can express a protein having high-affinity choline transporter activity according to any one of claims 34 to 38, or is the cell having high-affinity choline transporter activity according to claim 47 (claim 50), the screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to any one of claims 48 to 50, wherein the protein having high-affinity choline

transporter activity is a recombinant protein (claim 51), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell obtained from the non-human animal according to any one of claims 39 to 44 is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated (claim 52), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity (claim 53), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity (claim 54), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, and then evaluating the activity and/or the expression amount of

a protein having high-affinity choline transporter activity in comparison with the case using wild-type non-human animal (claim 55), and the screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to any one of claims 52 to 55, wherein the non-human animal is a mouse or a rat (claim 56).

The present invention further relates to a material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to any one of claims 48 to 56 (claim 57), a material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to any one of claims 48 to 56 (claim 58), a medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the protein according to any one of claims 14 to 22, and/or the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 57 as an active component (claim 59), and a medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the protein according to any one of claims 14 to 22, and/or the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 58 as an active component (claim 60).

The present invention still further relates to a diagnostic method for diseases relating to the expression or the activity of a high-affinity choline transporter characterized in comparing a DNA sequence encoding a high-affinity choline transporter in a sample to a DNA sequence encoding the protein according to claim 19 or 20 (claim 61), a diagnostic probe for Alzheimer's disease comprising a whole or a part of an antisense strand of DNA or RNA encoding the protein according to claim 19 or 20 (claim 62), and a diagnostic drug for Alzheimer's disease characterized in containing the diagnostic probe according to claim 62 and/or the antibody according to any one of claims 28 to 33 (claim 63).

Brief Explanation of the Drawings

Fig. 1 is a view showing the result of [^{3}H] choline uptake of oocytes from Xenopus of the present invention being injected with nematode cho-1 (C48D1.3 cRNA) or water.

Fig.2 is a view showing the result of the effect of Na^+ on choline uptake of oocytes from Xenopus of the present invention being injected with nematode cho-1 (C48D1.3 cRNA) or water.

Fig.3 is a view showing the result of the HC3-induced inhibition of choline uptake of oocytes from Xenopus of the present invention being injected with nematode cho-1 (C48D1.3 cRNA) or water.

Fig.4 is a view showing amino acid sequences of rat CHT1 and nematode CHO-1 of the present invention respectively.

Fig.5 is a view showing the distribution of neurons expressing cho-1::gfp of the present invention in the nervous system of nematode.

Fig.6 is a view showing the phylogenetic tree of Na^+-dependent glucose transporter family.

Fig.7 is a view showing an expected topology of rat CHT1 of the present invention.

Fig.8 is a view showing the result of Northern blot analysis of CHT1 mRNA transcript in rat tissue of the present invention.

Fig.9 is a view showing the result of *in situ* hybridization analysis of CHT1 transcript in a rat brain of the present invention.

Fig.10 is a view showing the result of *in situ* hybridization analysis of CHT1 transcript in a spinal cord of the present invention.

Fig.11 is a view showing the result of [^{3}H] choline uptake of oocytes from Xenopus of the present invention being injected CHT1 cRNA of the present invention or water.

Fig.12 is a view showing the effect of choline concentration on choline uptake in CHT1 of the present invention.

Fig.13 is a view showing the result of HC3-induced inhibition of choline uptake of CHT1 of the present invention.

Fig.14 is a view showing the result of Na^+- and Cl^--dependent choline uptake of CHT1 of the present invention.

Fig.15 is a view showing the result of [^{3}H] HC3 binding to the membrane prepared from COS7 cells being introduced with CHT1 cDNA of the present invention or vector pcDNA 3.1 separately.

Fig.16 is a view showing the result of saturation analysis of specific [^{3}H] HC3 binding to the membrane prepared from COS7 cells being introduced with CHT1 cDNA of the present invention

or vector pcDNA 3.1 separately.

Fig.17 is a view showing the result of displacement of specific [^{3}H] HC3 binding by HC3 of the present invention, choline (Cho), acetylcholine (ACh).

Best Mode for Carrying out the Invention

The cDNA of nematode high-affinity choline transporter of the present invention, being described in Seq. ID No. 1, can be obtained by injecting each cRNA prepared from candidate full-length cDNAs, which are expected as a member of Na^+-dependent transporter family according to C. elegans genome project, into oocytes of Xenopus, and examining the uptake of choline. The high-affinity uptake of choline in brain synaptosomes of mammals was completely inhibited by 1 μMhemicholinium-3 (HC3) (Ki=10-100 nM), while the low-affinity uptake of choline, which is distributed in every cells, was inhibited only by HC3 with higher concentration (Ki=50 μM). Therefore, the sensitivity to 1 μM HC3 can be used as criteria of high-affinity choline uptake during the process. For example, it is possible to confirm the identification, the expression, and the localization of an object gene from the candidate cDNA of a nematode (C. elegans) as follows.

It has been found that cDNA corresponding to the gene expected as C48D1.3 promotes significant choline uptake, being inhibited by 1 μM HC3, in the high-affinity choline uptake process. Fig. 1 shows the result of [^{3}H] choline uptake of oocytes from Xenopus being injected with C48D1.3 cRNA or water. In Fig. 1, the closed and the open columns indicate choline uptake in the absence or the presence of 1 μM HC3 respectively, and each column is shown by mean ± SEM (n=6 to 8 oocytes). Fig.2

shows the effect of Na^+ on the choline uptake, and the closed columns indicate choline uptake measured in the standard solution ([Na^+]=100 mM), the open columns indicate choline uptake in the absence of Na^+ (Na^+ was substituted with Li^+). In addition, Fig. 3 shows the inhibition of choline uptake induced by HC3. Based on the above-mentioned Fig. 2 and 3, it is presumed that the uptake is Na^+-dependent, and that Ki of HC3 is 50 nM. The cDNA clone was designated as cho-1 (high-affinity choline transporter-1).

By comparing a base sequence of cDNA and that of genome, cho-1 gene was found to comprise 9 exons. A protein expected from a base sequence of cDNA of cho-1 includes 576 amino acid residues (see Fig. 4), and this protein, being represented by Seq. ID No. 2, can be constructed by a usual method. When the available data base was searched, the amino acid sequences of cho-1 showed weak, but significant homology to members of Na^+-dependent glucose transporter family. Hydrophobic analysis and comparison to other transporters suggest that there is a twelve-transmembrane region (see Fig. 7).

Then, in order to identify cells expressing cho-1 in the nervous system of a nematode (C. elegans), a gene of a green fluorescent protein (GFP) fused with a region 5.1kb upstream from cho-1 gene was introduced into a nematode, and distribution of neurons expressing cho-1::gfp was examined. A photograph of L1 larva possessing cho-1::gfp reporter DNA at the outside of chromosome is shown as Fig. 5 (scale bar; 50 μm). In Fig. 5, the arrowhead indicates nerve ring. In the ventral nerve cord, GFP is expressed only in cholinergic motor nerve, however, some of DA, DB nerve cells do not express GFP owing probably to deficiency of reporter DNA at the outside of chromosome. It

supports the idea that cho-1 is a high-affinity choline transporter of the cholinergic neuron.

The cDNA of rat high-affinity choline transporter of the present invention, being described in Seq. ID No. 3, can be prepared, for example, by a method comprising the steps of: paying attention to cho-1 homologous molecules of vertebrates and searching data base with amino acid sequences expected from cho-1, and identifying one candidate (GenBank accession number: AQ 316435) in human genomic survey sequence (GSS); amplifying cDNA fragments from rat spinal cord cDNA by PCR with degenerate primers on the basis of homology of base sequences between the human genome DNA and cho-1; screening rat spinal cord cDNA library with this fragment, and a positive cDNA clone was obtained. A protein with 580 amino acid residues showing 51% identity and 70% similarity to cho-1 was expected from the base sequence of the longest reading frame (see Fig. 4). This rat cDNA clone was designated as CHT1. In Fig. 4, each amino acid sequence of rat CHT1 and nematode CHO-1 is shown, and the identical and the similar residues are indicated on a black ground and a gray ground respectively. The expected transmembrane region I-XII is underlined. This protein represented by Seq. ID No. 4 can be constructed by a usual method.

The above-mentioned amino acid sequence of CHT1 is significantly homologous to members of Na^+-dependent glucose transporter family (20 to 25%). The phylogenetic tree of Na^+-dependent glucose transporter family made by neighbor-joining method using a program CLUSTALW of National Institute of Genetics (Mishima, Japan) is shown in Fig. 6. In Fig. 6, the percentage of the identical amino acids, being contained in each protein, to rat CHT1 is shown on the right side. On

the other hand, no homology was observed to a yeast choline transporter (J. Biol. Chem. 265, 15996-16003, 1990), a creatine transporter which had been originally reported as a high-affinity choline transporter (Biochem. Biophys. Res. Commun. 198, 637-645, 1994), and other neurotransmitter transporters.

The expected topology of CHT1 is thought to be the same as that of nematode CHO-1 fundamentally. Fig. 7 shows the expected topology of rat CHT1. In Fig. 7, the closed circles indicate the identical residues, the shadowed circles indicate highly conserved residues, and open circles indicate nonsimilar residues. The offshoots indicate the expected glycosylation sites. P among the circles shows the expected parts of phosphorylation induced by protein kinase C.

Next, the distribution of CHT1 mRNA expression was examined by Northern blot analysis and *in situ* hybridization. The expression of transcripts with the length of about 5 kb was confirmed by Northern blot analysis of various tissues of rats. Fig. 8 shows the result of Northern blot analysis of mRNA transcript of CHT1 in rat tissue, and the length of RNA standard (0.24 to 9.5 kb; GIBCO BRL) is exhibited on the left side. As shown in Fig. 8, an abundance of transcripts were confirmed in basal forebrain, brain stem and spinal cord, and a little of those were confirmed in corpus striatum. These tissues are known to contain cholinergic neurons. On the other hand, no transcript was observed in other regions of the brain or in tissues of non-nervous systems.

Consistent with these results, *in situ* hybridization confirmed the expression of CHT1 mRNA in cell groups of main cholinergic neurons including corpus striatum, cell population in basal forebrain and ventral horn in spinal cord. Fig. 9 and

10 (scale bar; 1 mm) show micrographs of sections in bright-field, which were hybridized with a cRNA probe of an antisense labeled by digoxigenin. These micrographs relate to in situ hybridization analysis of CHT1 transcripts in rat brain and spinal cord. Fig. 9 indicates that mRNA transcripts of CHT1 were detected in vertical and horizontal limbs of the diagonal band (VDB, HDB), medial septal nucleus (MS), caudate and putamen (Cpu), and olfactory tubercle (Tu). Fig. 10 indicates that the expression was observed in ventral horn (VH) in spinal cord. Further, the adjacent section hybridized with a probe of vesicle acetylcholine transporter showed essentially same distribution. This expression distribution is essentially same as the reported distribution of cholineacetyl group transferase or vesicle acetylcholine transporter. These results show that the expression of CHT1 mRNA is limited to cholinergic neurons.

Next, choline uptake of CHT1 was examined by using oocytes of Xenopus. The choline uptake of the oocytes injected with CHT1 cRNA was 2 times to 4 times more than that of controls injected with water. Fig. 11 shows the result of [^{3}H] choline uptake of oocytes of Xenopus injected with CHT1 cRNA or water. In Fig. 11, the open and the closed columns respectively indicate choline uptake in the standard solutions containing 100 mM NaCl or LiCl, and each column is shown by mean ± SEM (n=6 to 8 oocytes). The effect of choline concentration on choline uptake is shown in Fig. 12. In Fig. 12, choline uptake of oocytes injected with water was subtracted from that of oocytes injected with cRNA in order to figure out CHT1-induced choline uptake, and the choline uptake was fitted to Michaelis-Menten curve. As shown in Fig. 12, choline uptake of CHT1 saturated

when increasing choline concentration (Km=2.2 ± 0.2 μM, n=3). The Km of endogenous choline uptake of control is higher than 10 μM.

Then, the result of HC3-induced inhibition of choline uptake is shown in Fig. 13. Fig. 13 indicates that choline uptake of CHT1 is completely inhibited by 0.1 μM HC3 (Ki=2-3 nM), whereas 10 μM HC3 induced only slight inhibition in control. As shown in Fig. 14, ion-dependency of choline uptake of CHT1 was examined and found to be Cl^--dependent as well as Na^+-dependent. The closed and the open columns indicate choline uptake of oocytes injected with water and with cRNA respectively (100 mM NaCl in the standard solution is substituted with 100 mM of each salt) shown in the figure. These results indicate that CHT1 has the characteristics expected from high-affinity choline uptake in brain synaptosomes (high-affinity to choline, high sensitivity to HC3, and Na^+-Cl^--dependency) (J. Neurochem. 27, 93-99, 1976).

In addition, [^{3}H] HC3 binding activity of membranes prepared from COS7 cells introduced with CHT1 cDNA and a vector (control) respectively was examined. The result is shown in Fig. 15. As Fig. 15 indicates, Na^+-dependent [^{3}H] HC3 binding was observed in a membrane of a cell where CHT1 was expressed, but not in a control membrane. Subsequently, a saturation analysis was conducted for specific [^{3}H] HC3 binding. As shown in Fig. 16, equilibrium dissociation constant (Kd) was estimated to be 1.6±0.2μM (n=3). This value was similar to that reported in brain synaptosomes (J. Neurochem. 60, 1191-1201, 1993, Life Sci. 35, 2335-2343, 1984, Brain Res. 348, 321-330, 1985). Further, displacement of specific [^{3}H] HC3 binding by HC3, choline (Cho) and acetylcholine (Ach) was examined.

Acetylcholine was measured in the presence of 1 μM physostigmine. The result is shown in Fig. 17. Fig. 17 indicates that specific [^{3}H] HC3 binding was displaced when the concentration of choline was at least about 10 times lower than that of acetylcholine. These results show that CHT1 is a HC3 binding site as well as a high-affinity choline transporter.

The cDNA of human high-affinity choline transporter of the present invention, being represented by Seq. ID No.5, can be prepared, for example, as follows: data base search was conducted with the amino acid sequence of nematode (C. elegans) CHO-1 to find a sequence of specific human genome DNA fragment having significant homology (R-107P12, a clone of human genomic survey sequence; GenBank accession number: AQ316435); a gene-specific primers for PCR were designed based on a base sequence of said DNA fragment; 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE were conducted using Marathon-Ready™ cDNA (Clontech) of human whole brain, together with an attached adapter primer; the obtained PCR product was cloned into a cloning vector for PCR, and a base sequence of inserted DNA was determined. In addition, an amino acid sequence expected from this DNA sequence is represented by Seq. ID No. 6. A protein having human high-affinity choline transporter activity represented by said Seq. ID No. 6 can be constructed by a usual method on the basis of DNA sequence information shown in Seq. ID No. 5.

The cDNA of mouse high-affinity choline transporter of the present invention, being represented by Seq. ID No.7, can be prepared, for example, as follows: data base search was conducted with the amino acid sequence of nematode (C. elegans) CHO-1 to find a sequence of specific human genome DNA fragment

having significant homology (R-107P12, a clone of human genomic survey sequence; GenBank accession number: AQ316435); a gene-specific primer for PCR was designed based on a base sequence of said DNA fragment; 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE were conducted using Marathon-Ready™ cDNA (Clontech) of mouse whole brain, together with an attached adapter primer; the obtained PCR product was cloned into a cloning vector for PCR, and a base sequence of inserted DNA was determined. In addition, an amino acid sequence expected from this DNA sequence is represented by Seq. ID No. 8. A protein having mouse high-affinity choline transporter activity represented by said Seq. ID No. 8 can be constructed by a usual method on the basis of DNA sequence information shown in Seq. ID No. 7.

Examples of a protein having high-affinity choline transporter activity of the present invention include a protein derived from natural materials and a recombinant protein. In addition to the ones represented by Seq. ID Nos. 2, 4, 6 and 8, which are specifically disclosed above, a protein comprising an amino acid sequence wherein one or a few amino acids are deficient, substituted or added in amino acid sequences represented by Seq. ID Nos. 2, 4, 6 and 8, and having high-affinity choline transporter activity is also included. These proteins can be prepared by known methods. Further, examples of a gene or DNA encoding a protein having high-affinity choline transporter activity of the present invention include, in addition to the ones represented by Seq. ID Nos. 1, 3, 5 and 7, which are specifically disclosed above, a gene or DNA which encodes a protein comprising an amino acid sequence wherein one or a few amino acids are deficient, substituted or added in amino

acid sequences represented by Seq. ID Nos. 2, 4, 6 and 8, and having high-affinity choline transporter activity, and DNA which encodes a protein hybridizing with said gene or DNA under a stringent condition and having high-affinity choline transporter activity. These genes and DNAs can be prepared by known methods.

Cholinergic neurons play an extremely important role in learning and memory. The damage of these neurons correlates to severity of dementia. The rate-limiting step in acetylcholine synthesis is presumed to be the uptake of choline, and its activity is controlled by neural activity or various kinds of stimuli. In the brains of patients who suffer Alzheimer's disease, the hyperfunction of high-affinity choline uptake and of HC3 binding activity are observed (Trends Neurosci. 15, 117-122, 1992, Ann. NY Acad. Sci. 777, 197-204, 1996, J. Neurochem. 69, 2441-2451, 1997). Cloning of said gene or DNA encoding a protein having high-affinity choline transporter activity and said protein having high-affinity choline transporter activity is important for elucidating the molecular mechanism of the high-affinity choline transporter and for developing new therapies for Alzheimer's disease.

The fusion protein of the present invention means a substance constructed by binding a protein from a nematode, a rat, a human, a mouse, etc., which has high-affinity choline transporter activity, to a marker protein and/or a peptide tag. As the marker protein, any conventionally known marker protein can be used and the specific examples are alkaline phosphatase, Fc region of an antibody, HRP, and GFP. Conventionally known peptide tags, such as Myc tag, His tag, FLAG tag, GST tag, are exemplified as specific examples of the peptide tag of the

present invention. Said fusion proteins can be constructed by a usual method, and are useful for the purification of a protein having high-affinity choline transporter activity utilizing the affinity between Ni-NTA and His tag, the detection of a protein having high-affinity choline transporter activity, the quantitation of an antibody to a protein having high-affinity choline transporter activity, and as a diagnostic marker for Alzheimer's disease, and an investigational reagent in the field concerned.

As an antibody that specifically combines with a protein having high-affinity choline transporter activity of the present invention, an immunospecific antibody such as a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a single stranded antibody, a humanized antibody and the like are concretely exemplified. Though these antibodies can be constructed by a usual method with the above-mentioned protein having high-affinity choline transporter activity as an antigen, a monoclonal antibody is more preferable among them because of its specificity. Said antibody that specifically binds to a protein having high-affinity choline transporter activity, such as a monoclonal antibody or the like, is useful, for instance, for the diagnosis of Alzheimer's disease, and for elucidation of molecular mechanism of a high-affinity choline transporter.

An antibody to a protein having high-affinity choline transporter activity is developed by administering fragments containing the protein having high-affinity choline transporter activity or its epitope, or cells that express said protein on the surface of the membrane, to animals (preferably excluding human) with usual protocol. For instance, a

monoclonal antibody can be prepared by an arbitrary method that brings antibodies developed by cultured materials of continuous cell line, such as hybridoma method (Nature 256, 495-497, 1975), trioma method, human B-cell hybridoma method (Immunology Today 4, 72, 1983), and EBV-hybridoma method (MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp. 77-96, Alan R. Liss, Inc., 1985).

In order to develop a single stranded antibody to the above-mentioned protein having high-affinity choline transporter activity of the present invention, the preparation method of single stranded antibodies (US Patent No. 4,946,778) can be applied. Further, in order to express a humanized antibody, it is possible to use transgenic mice, other mammalian animals or the like, and to isolate and identify the clones that express a protein having high-affinity choline transporter activity with the above-mentioned antibodies, and to purify the polypeptide by affinity chromatography. An antibody to a protein having high-affinity choline transporter activity could be used, in particular, for the diagnosis and the medical treatment of Alzheimer's disease, and the like.

This invention relates to a host cell which contains an expression system that can express said protein having high-affinity choline transporter activity. The gene that encodes a protein having high-affinity choline transporter activity can be introduced into a host cell by a number of methods described in many standard laboratory manuals such as by Davis et al. (BASIC METHODS IN MOLECULAR BIOLOGY, 1986), and by Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989). Examples of those methods include calcium phosphate transfection, DEAE-dextran-mediated transfection,

transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection. Examples of the host cells include bacterial procaryotic cells such as Escherichia coli, Streptomyces, Bacillus subtilis, Streptococcus, Staphylococcus and the like; fungous cells such as yeast, Aspergillus and the like; insect cells such as drosophila S2, spodptera Sf9 and the like; and animal or plant cells such as L cells, CHO cells, COS cells, HeLa cells, C127 cells, BALB/c3T3 cells (including mutant strains deficient in dihydrofolate reductase, thymidine kinase or the like), BHK21 cells, HEK293 cells, Bowes melanoma cells and the like.

As the expression system, any expression system that can express a protein having high-affinity choline transporter activity in a host cell will suffice. Examples of the expression system include expression systems derived from chromosome, episome and virus, for example, vectors derived from bacterial plasmid, yeast plasmid, papovavirus like SV40, vaccinia virus, adenovirus, chicken pox virus, pseudorabies virus, or retrovirus, vectors derived from bacteriophage, transposon, and the combination of these, for instance, vectors derived from genetic factors of plasmid and of bacteriophage such as cosmid or phagemid. These expression systems may contain a regulatory sequence that acts not only as a promoter but also as a controller of expressions.

A host cell that contains the above-mentioned expression system, cell membrane of said host cell, and a protein having high-affinity choline transporter activity which is obtainable by the cultivation of said host cell can be used in the screening method of the present invention as hereinafter described. For

example, the method of F. Pietri-Rouxel et al. (Eur. J. Biochem., 247, 1174-1179, 1997) or the like can be used as the method to obtain cell membranes, and publicly known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic-interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin chromatography, preferably high-speed liquid chromatography can be used to pick up said protein having high-affinity choline transporter activity from cell cultured material and purify it. As columns used for affinity chromatography, in particular, there are columns to which a protein antibody having anti-high-affinity choline transporter activity is bound, or in case that a normal peptide tag is added to said high-affinity choline transporter, there are columns to which materials having affinity to the peptide tag are bound. Proteins having high-affinity choline transporter activity can be obtained by using these columns.

In the present invention, said non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient on its chromosome means a non-human animal wherein a part or a whole of a gene encoding a protein having high-affinity choline transporter activity on chromosome is inactivated by gene mutation such as disruption, deficiency, substitution, etc. and function of expressing a protein having high-affinity choline transporter activity is lost. In addition, a non-human animal that overexpresses function of a gene that encodes a protein having high-affinity choline transporter activity on its chromosome means a non-human animal that produces larger amount of a

protein having high-affinity choline transporter activity than a wild-type non-human animal does. Though specific examples of a non-human animal of the present invention include rodents, such as mice, rats and the like, a non-human animal of the present invention is not limited to these animals.

Homozygous non-human animals generated according to Mendelian ratio include a deficient type or an overexpression type for a protein having high-affinity choline transporter activity, and their littermate wild-type, and it is possible to carry out precise comparative experiments in individual level by using the deficient types, the overexpression types and the littermate wild-types of these homozygous non-human animals at the same time. Therefore, it is preferable to use animals of the same species, more preferably the littermates, as the wild-type non-human animals, in other words, the non-human animals being deficient in or overexpressing the function of a gene that encodes a protein having high-affinity choline transporter activity on their chromosome together in, for example, the screening hereinafter described in the present invention. The generating method of the non-human animals being deficient or overexpressing the function of a gene that encodes a protein having high-affinity choline transporter activity on their chromosome will be explained below, with an example of knockout mice and transgenic mice of a protein having high-affinity choline transporter activity.

For example, a mouse being deficient in the function of a gene that encodes a protein having high-affinity choline transporter activity on its chromosome, in other words, a knockout mouse of a protein having high-affinity choline transporter activity on its chromosome can be constructed as

follows. A gene that encodes a protein having high-affinity choline transporter activity is screened by using a gene fragment obtained from mouse gene library by a method like PCR. The screened gene that encodes a protein having high-affinity choline transporter activity is subcloned with a viral vector or the like, and specified by DNA sequencing. A target vector is constructed by substituting a whole or a part of a gene of this clone that encodes a protein having high-affinity choline transporter activity with pMC1 neo gene cassette or the like, and by introducing a diphteria toxin A fragment (DT-A) gene, a herpes simplex virus thymidine kinase (HSV-tk) gene or other such genes into 3'-terminal side.

This constructed target vector is linearized and introduced into ES cells by electroporation or the like to induce homologous recombination. The ES cells wherein homologous recombination is induced by an antibiotic such as G418, ganciclovir (GANC) or the like are selected from the homologous recombinants. It is preferable to confirm whether the selected ES cells are the recombinants of the object by Southern blot or the like. A chimeric mouse is constructed by microinjecting a clone of the confirmed ES cells into a blastocyst of a mouse and then transplanting the blastocyst into a recipient mouse. A heterozygous mouse can be obtained by interclossing the chimeric mouse with a wild-type mouse, and a knockout mouse of a protein having high-affinity choline transporter activity of the present invention can be constructed by interclossing the heterozygous mice. It is possible to confirm whether a knockout mouse of a protein having high-affinity choline transporter activity is constructed, for example, by isolating RNA from the mouse obtained by said method

and examining it by Northern blot analysis or the like, or by examining the expression of the mouse by Western blot analysis or the like.

The transgenic mice of a protein having high-affinity choline transporter activity can be generated in following procedures. A transgene is constructed by fusing chicken β-actin, mouse neurofilament, SV40 or other such promoters, and rabbit β-globin, SV40 or other such poly A or introns with cDNA that encodes a protein having high-affinity choline transporter activity. The transgene is microinjected into the pronucleus of a fertilized egg of a mouse, and the obtained egg cell is cultured, then transplanted to the oviduct of a recipient mouse. After rearing up the recipient animal, baby mice that have the above-mentioned cDNA are selected from the mice born from the recipient animal. Thus transgenic mice can be generated. The baby mouse that has cDNA can be selected by extracting crude DNA from a tail or the like of a mouse, then carrying out methods like dot hybridization using the introduced gene that encodes a protein having high-affinity choline transporter activity as a probe, PCR method using a specific primer, and the like.

In addition, cells being useful for gene therapy of Alzheimer's disease and the like can be prepared by using a whole or a part of a gene or DNA that encodes a protein having high-affinity choline transporter activity of the present invention. As an example of a method for preparing these cells of the present invention, a method wherein a whole or a part of said gene or DNA of the present invention is introduced into a cell being deficient in the function of a gene that encodes a protein having high-affinity choline transporter activity on its chromosome by transfection or the like to obtain a cell

having high-affinity choline transporter activity is exemplified. As the cell having high-affinity choline transporter activity, in particular, it is preferable to use a cell wherein said gene or DNA is integrated into a chromosome and high-affinity choline transporter activity is exhibited stably.

By using the above-mentioned gene or DNA that encodes a protein having high-affinity choline transporter activity, a protein having high-affinity choline transporter activity, a fusion protein created by combining a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, an antibody to a protein having high-affinity choline transporter activity, a host cell which contains an expression system that can express a protein having high-affinity choline transporter activity, a cell having high-affinity choline transporter activity, or the like, it becomes possible to screen a pharmaceutical material useful for the treatment of symptoms as in Alzheimer's disease or the like, in other words, a material that promotes or suppresses the activity or the expression of a high-affinity choline transporter.

Examples of the screening method of the present invention are: a method wherein the high-affinity choline transporter activity of the above-mentioned protein having high-affinity choline transporter activity of the present invention is measured/evaluated in the presence of a subject material; a method wherein a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity of the present invention is cultivated in vitro in the presence of a subject material, and the activity and/or the expression

amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated; and a method wherein a subject material is administered to said non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome and/or a wild-type non-human animal and then the activity and/or the expression amount of a protein having high-affinity choline transporter activity of the present invention is measured/evaluated. As said cell membrane or said cell, a cell such as a primary cultured cell obtained from said non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome or a wild-type non-human animal etc., a host cell containing an expression system which can express a protein having high-affinity choline transporter activity of the present invention, a cell having high-affinity choline transporter activity of the present invention, and cell membranes of these cells can be specifically exemplified.

The screening methods with said subject material and said protein having high-affinity choline transporter activity are now specifically explained together with examples, but the screening methods of the present invention are not limited to these examples. Cells expressing a protein having high-affinity choline transporter activity are cultured in the presence of a subject material, and the increase or the decrease of a protein having high-affinity choline transporter activity expressed on the cell surface after a certain period of cultivation can be immunochemically detected by ELISA or other such method with an antibody that specifically combines to a protein having high-affinity choline transporter activity of

the present invention, or can be evaluated by using suppression or promotion of mRNA expression as an index. The mRNA can be detected by methods such as DNA chip, Northern hybridization or the like. Moreover, with a cell to which a gene wherein luciferase or other such reporter genes is linked to downstream of promoter of a gene that encodes high-affinity choline transporter is introduced, the suppression or the promotion of the expression of a gene that encodes a protein having high-affinity choline transporter activity induced by a subject material can be detected by using the activity of said reporter gene as an index.

The present invention further relates a medical constituent being used for medical treatment for a patient who needs promotion of the activity or the expression of a protein having high-affinity choline transporter, or a medical constituent being used for medical treatment for a patient who needs suppression of the activity or the expression of a protein having high-affinity choline transporter, wherein the material contains a protein having high-affinity choline transporter activity, a material which promotes the activity or the expression of a protein having high-affinity choline transporter activity, or a material which suppresses activity or expression of a protein having high-affinity choline transporter activity as an active component. As a protein having high-affinity choline transporter activity is involved in many biological functions including many pathological ones, it is expected that a compound that can stimulate a protein having high-affinity choline transporter activity and a compound being able to inhibit the function of said protein can be used as pharmaceuticals.

As the material which promotes or suppresses the activity or the expression of a protein having high-affinity choline transporter activity, any material can be used as long as it binds to a protein having high-affinity choline transporter activity, or works on a signal transmitting molecule on upstream, and then promotes the activity or the expression of a protein having high-affinity choline transporter activity or inhibits/antagonizes the activity or the expression of the protein by itself. Specific examples include an antibody, a ligand of a protein having high-affinity choline transporter activity, a fragment of said protein, and an oligonucleotide encoding said fragment, and these materials can be used as pharmaceuticals for treatment, prevention or the like of symptoms observed in the case of Alzheimer's disease or other such diseases, but use of them is not limited to the above examples.

The present invention also relates to a diagnostic method for diseases relating to the activity or the expression of a protein having high-affinity choline transporter activity comprising a comparison of a DNA sequence encoding a protein having high-affinity choline transporter activity in a sample with a DNA sequence encoding a protein having high-affinity choline transporter activity of the present invention. The mutant type of DNA which encodes a protein having high-affinity choline transporter activity can be detected by finding gene-mutated individuals in DNA level, and this is useful for diagnosis of diseases caused by underexpression, overexpression or mutant expression of a protein having high-affinity choline transporter activity. Specific examples of a sample of said detection include cells of trial

subjects, for example, genomic DNA, RNA or cDNA obtained from biopsy of blood, urine, saliva, tissue or the like, however said sample is not limited to these examples. It is also possible to use said sample being amplified by PCR or other such methods. Deficiency and insertion mutation of base sequences can be detected by the size change of the amplified product observed in comparison with normal genotype, and point mutation can be identified by hybridizing amplified DNA with a labeled gene that encodes a protein having high-affinity choline transporter activity. Thus, diagnosis or judgement of symptoms observed in the case of Alzheimer's disease or other such diseases can be made by detecting the mutation of a gene that encodes a protein having high-affinity choline transporter activity.

The present invention further relates to a diagnostic probe for diseases showing symptoms similar to those of Alzheimer's disease or the like comprising a whole or a part of an antisense chain of DNA or RNA encoding a protein having high-affinity choline transporter activity, and a diagnostic drug for diseases showing symptoms similar to those of Alzheimer's disease containing the diagnostic probe and/or an antibody which specifically binds to a protein having high-affinity choline transporter activity of the present invention. Said diagnostic probe is not limited in particular, as long as it comprises a whole or a part of an antisense chain of DNA (cDNA) or RNA (cRNA) encoding a protein having high-affinity choline transporter activity and being long enough to be a probe (at least 20 bases). In order to make a diagnostic drug for symptoms similar to those of Alzheimer's disease containing said probe and/or an antibody which specifically binds to a protein having high-affinity choline transporter activity of the present

invention as active components, it is preferable to dissolve said probe into an appropriate buffer or sterilizing water for preventing said probe from decomposition. Further, it is also possible to diagnose diseases showing symptoms similar to those of Alzheimer's disease by methods using these diagnostic drugs, such as immunostaining (Dev. Biol. 170, 207-222, 1995, J. Neurobiol. 29, 1-17, 1996), in situ hybridization (J. Neurobiol. 29, 1-17, 1996), in situ PCR or the like.

Experimental methods or the like of the above-mentioned various experiments will now be explained in more detail below.

(Cloning of high-affinity choline transporter cDNA)

The candidate cDNA of nematode high-affinity choline transporter was isolated from poly (A)+RNA of nematode mixture from various stages in the development by reverse transcription PCR and 3' RACE. Marathon™ cDNA Amplification Kit (Clontech) was used according to its protocol. A primer for sense direction of PCR was designed at a provisional translation initiating point of a predicted gene based on a DNA base sequence obtained from C. elegans genomic project. The amplified PCR product was subcloned into Nco I (smoothing) site and Not I site of a modified pSPUTK vector (Stratagene), and the base sequence of inserted DNA was determined. CHT1 cDNA of rat was isolated from rat spinal cord cDNA library by using GeneTrapper cDNA Positive Selection System (GIBCO Bio-Rad Laboratory: GIBCO BRL) according to its protocol. The primer used was designed from the base sequence of a cDNA fragment obtained by degenerated PCR. The obtained cDNA clones were analyzed. Among them, positive clones were selected and subcloned into pSPUTK vector and pcDNA3.1+ vector (Invitrogen Corporation).

(Expression in oocytes of Xenopus)

In the presence of cap analog, cRNA was synthesized in vitro with SP6 or T7 RNA polymerase. 20 to 30 ng capped RNA was microinjected into oocytes (stage V to VI) of Xenopus. The uptake was measured in basically same manner as described previously (Nature 360, 467-471, 1992). Two or three days after the injection of RNA, choline uptake was conducted for 30 to 60 min. with oocytes (6 to 8) in 0.75 ml standard solution (0.01 to 1 μM [^{3}H]-choline, 100 mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 5mM Tris: pH 7.4). The oocytes completing uptake were solubilized with 10% SDS, and the amount of [^{3}H] was measured by a liquid scintillation counter.

(GFP expression construct)

The transcriptional fusion construct of cho-1::gfp was constructed by PCR in same manner as described previously (Gene 212, 127-135, 1998). A gene that encodes a green fluorescent protein (GFP) located on downstream of a nuclear localization signal sequence (NLS) was inserted into a position 3 residues downstream of cho-1 translation initiating point so that the reading frame was fitted. NLS and gfp gene were amplified from pPD104.53 vector. In order to prepare 5.1 kb upstream region of cho-1 translation initiating point, a PCR primer being designed to encompass the first 3 amino acid residues of cho-1 was used. By the same method as previously described (EMBO J. 10, 3959-3970, 1991), rol-6 (su1006) marker and generated DNA were injected into gonads of a nematode simultaneously.

(Northern blot analysis)

6 µg poly(A)+RNA prepared from various tissues of rats was separated by formaldehyde-agarose electrophoresis, and transferred to a nylon membrane, then hybridized with CHT1 cDNA fragment being labeled with [^{32}P] by random prime method in hybridization solution (solution containing the final concentration of 50% formamido, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 µg/ml salmon sperm DNA) at 42° C for 16 hours. The nylon membrane was washed under final condition (0.1 × SSPE, 0.1% SDS: 65° C), and then autoradiography was conducted for 7 days together with an enhancing screen.

(In situ hybridization)

The transcript of an antisense labeled with digoxigenin was synthesized in vitro. Alkaline hydrolysis was repeated for the transcripts until their mean length was prepared to be 200 to 400 b. Cryostat sections of fresh frozen tissue (10 to 20 µm) were used. Hybridization was conducted with labeled cRNA probe (about 1 µg/ml) dissolved in 1 × Denhardt's solution [solution containing the final concentration of 50 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.3 M NaCl, 50% formamido, 10% dextran sulphate, 1 mg/ml E. coli tRNA] at 45° C for 20 hours. Then the sections were washed twice in 2 × SSC/50% formamido and once in 1 × SSC/50% formamido, at 45° C respectively. The hybridized probe was visualized by using anti-digoxigenin Fab fragment (Boehringer-Mannheim) and NBT/BCIP substrate. The sections were brought into reaction in substrate solution for 24 to 48 hours.

(Binding assay)

[^{3}H] hemicholinium-3 (HC3; 128Ci/mmol) was obtained from

NEN Life Science Products. Either pcDNA3.1-CHT1 or pcDNA3.1 was transiently expressed in COS7 cells respectively. TransFast Reagent (Promega) was introduced and used according to the protocol. Membranes were prepared by following steps: homogenizing cells in 0.32 M sucrose; centrifuging the cells for 1 hour at 200,000g; and suspending the precipitate. Binding assay was conducted in basically same manner as described previously. Specific binding amount was calculated by subtracting non-specific binding amount determined in the presence of 10 μM HC3 from the whole binding amount. The Kd value was figured out by analyzing specific [^{3}H] HC3 binding amount from data of saturation binding assay with nonlinear approximation.

Industrial Applicability

The present invention makes it possible to provide a protein having high-affinity choline transporter activity, which is physiologically important, and gene DNA encoding said protein. In addition, by using the said protein and gene DNA, it becomes possible to screen materials being useful for prevention or treatment of Alzheimer's disease, and to prepare cells being useful for gene therapy.